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The interaction between human blood-coagulation Factor VIII and von Willebrand Factor

Characterization of a high-affinity binding site on Factor VIII

Anja LEYTE,* Martin Ph. VERBEET,* Teresa BRODNIEWICZ-PROBA,† Jan A. VAN MOURIK*‡ and Koen MERTENS*

*Department of Blood Coagulation, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, 1006 AK Amsterdam, The Netherlands, and †Centre de Fractionnement Sanguin 'Armand Frappier', Laval, Quebec H7V 1B7, Canada

The interaction between human Factor VIII and immobilized multimeric von Willebrand Factor (vWF) was characterized. Equilibrium binding studies indicated the presence of multiple classes of Factor VIII-binding sites on vWF. The high-affinity binding ($K_d = 2.1 \times 10^{-10}$ M) was restricted to only 1–2% of the vWF subunits. Competition studies with monoclonal antibodies with known epitopes demonstrated that the Factor VIII sequence Lys^{1673}–Arg^{1689} is involved in the high-affinity interaction with vWF.

INTRODUCTION

The complex between blood-coagulation Factor VIII and von Willebrand Factor (vWF) serves an essential role in the haemostatic process [1]. Factor VIII functions as a cofactor in the activation of Factor X in the intrinsic coagulation pathway [2–4]. The single polypeptide chain of M_r 260000 [5,6] is susceptible to limited proteolysis, and consequently circulates as heterodimers of a *C*-terminal light chain (M_r 80000) and variable derivatives (M_r 50000–180000) of the *N*-terminal heavy chain [7–9]. The persistence of Factor VIII in the circulation is strongly dependent on complex-formation with vWF [1,10,11]. vWF circulates in plasma as a heterogeneous population of multimerized dimeric forms [1,12]. These dimers are composed of monomeric subunits of M_r 250000 that are disulphide-linked in their *C*-terminal regions; disulphide linkage between the free *N*-termini then results in multimerization [1,13]. A minor proportion of the vWF subunits in the multimers contains an uncleaved *N*-terminal pro-sequence of M_r 100000 [1,14].

The nature of the interaction between vWF and Factor VIII is poorly documented. On vWF, a major Factor VIII-binding domain has been located within amino acid residues 1–272 of the mature subunit [15]. The Factor VIII light chain has been shown to be involved in vWF binding [16]. In the present study we describe a method for studying the binding of Factor VIII to vWF. Data are presented on the characteristics of the interaction and on the fine mapping of the vWF-binding site on the Factor VIII light chain.

MATERIALS AND METHODS

Purified proteins

Human Factor VIII was purified by a method devised by A. Leyte, M. J. M. de Keyzer-Nellen, M. M. C. L. Groenen-van Dooren, J. de Bruin, H. Pannekoek, J. A. Van Mourik & M. Ph. Verbeet (unpublished work). In summary, Factor VIII concentrate (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service) was dissolved in a buffer containing 0.25 M-$CaCl_2$ to dissociate the Factor VIII–vWF complex. Factor VIII then was isolated by affinity chromatography using a monoclonal antibody against Factor VIII (CLB-CAg 117; see ref. [17]). Purified Factor VIII obtained by this method had a ratio of Factor VIII activity over antigen of 0.9, had a specific activity of 2500–3000 units/mg, and contained less than 0.1% (w/w) vWF. Human vWF was prepared from the same source, by gel filtration of the Factor VIII-depleted concentrate over Bio-Gel A-5m (Bio-Rad Laboratories, Richmond, CA, U.S.A.) in 150 mM-NaCl/50 mM-imidazole/HCl buffer, pH 6.8. The void-volume fractions were pooled and the vWF was concentrated by $(NH_4)_2SO_4$ precipitation (35% saturation, at 4 °C), and dissolved in 150 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.4. The specific vWF antigen content of the final preparation was 100 units/mg. Less than 0.1% of the total protein was residual Factor VIII. The material migrated as a single band (M_r 250000) on SDS/polyacrylamide-gel electrophoresis under reducing conditions. Multimer analysis demonstrated the presence of vWF multimeric forms with M_r values up to at least 5×10^6 (cf. Fig. 1).

Monoclonal antibodies

Mouse monoclonal antibodies against human Factor VIII were produced and purified as described previously [18]. The epitope of antibody CLB-CAg 69 was mapped on the same region of the Factor VIII light chain (amino acid residues 1649–1778) as reported previously for the antibody CLB-CAg 65 [19]. The control antibody CLB-CAg 9 recognizes an epitope located between residues 712 and 741 on the heavy chain of Factor VIII [19]. These antibodies do not inhibit Factor VIII activity when tested in the spectrophotometric Factor VIII assay (see below).

Abbreviation used; vWF, von Willebrand Factor.

‡ To whom correspondence should be addressed: c/o Publication Secretariat, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, P.O. Box 9406, 1006 AK Amsterdam, The Netherlands.

Synthetic Factor VIII peptide

The synthetic peptide representing the Factor VIII sequence Lys^{1673}–Arg^{1689}, preceded by Cys-Gly- at the *N*-terminus, was provided by Dr. M. Lacroix, Centre de Fractionnement Sanguin 'Armand Frappier'.

Factor VIII–vWF binding assay

Micro-titre wells (Immulon; Dynatech G.m.b.H., Plockingen, Germany) were coated with vWF, as follows: various amounts (0–50 munits) of purified human vWF were incubated in 150 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.4 (100 μl/well), for 2 h at 37 °C. The wells were washed once with 250 mM-$CaCl_2$/150 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.4, to eliminate any traces of Factor VIII, and twice with the same buffer without $CaCl_2$. Subsequently, the wells were incubated with, per well, 100 μl of 2% (w/v) human serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.)/0.1% (w/v) Tween 20 (Merck, Darmstadt, Germany) in 150 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.4, for 1 h at 37 °C or overnight at 4 °C. After this blocking the wells were washed three times with the same buffer containing 0.2% (w/v) human serum albumin. The amounts of vWF immobilized by this protocol were determined by the use of radiolabelled [20] vWF as a tracer, and ranged from 0.7 to 5 munits of vWF per well (about 10% of the input). The vWF-coated wells were incubated with various amounts of Factor VIII (0–370 munits) in 100 μl of 2% (w/v) human serum albumin/0.1% (w/v) Tween 20 in 2.5 mM-$CaCl_2$/150 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.4, for 2 h at 37 °C. Non-bound Factor VIII was removed by two immediately successive washes with the same buffer containing 0.2% (w/v) human serum albumin. Dissociation studies (see Fig. 1) demonstrated that this rapid washing procedure (less than 5 s) did not disturb the binding equilibrium. Bound Factor VIII was then quantified spectrophotometrically (see below), with the purified Factor VIII preparation as reference.

Quantification of proteins

Concentrations of total protein were determined by the method of Bradford [21]. vWF antigen was quantified as described previously [22]. Factor VIII light chain was measured by using an enzyme-linked immunosorbent assay based on a previously described method [17]. Factor VIII activity was determined spectrophotometrically, by using a method employing a chromogenic substrate and purified bovine coagulation factors (Coatest Factor VIII; KabiVitrum, Stockholm, Sweden). The assay was performed in micro-titre wells essentially as prescribed by the manufacturer. Factor VIII and vWF concentrations are expressed in units/ml; this represents the concentration of activity or antigen in 1 ml of pooled normal human plasma. One unit of Factor VIII and 1 unit of vWF are approximately equivalent to 0.1 μg and 10 μg of protein respectively [1].

RESULTS AND DISCUSSION

In the present paper we describe a method for studying the interaction between Factor VIII and vWF. The assay system differs from previously described methods also employing imobilized vWF [16,23,24] in that bound Factor VIII is quantified by its biological activity by using a spectrophotometric assay. This approach is based on the notion that the presence of vWF has no effect on the cofactor function of activated Factor VIII [3,25] and allows expression of specific binding of Factor VIII in quantitative terms. Control experiments demonstrated that Factor VIII does not bind to micro-titre wells lacking vWF coating. Factor VIII binding to multimeric vWF was found to be maximal after 1 h of incubation, and could be reversed by the addition of $CaCl_2$ (final concentration 0.25 M), a condition known to dissociate the Factor VIII–vWF complex [1]. Reversibility of binding was further studied with dissociation experiments in the absence and in the presence of multimeric vWF in solution (Fig. 1). In the absence of vWF, dissociation occurred with an apparent $t_{\frac{1}{2}}$ of about

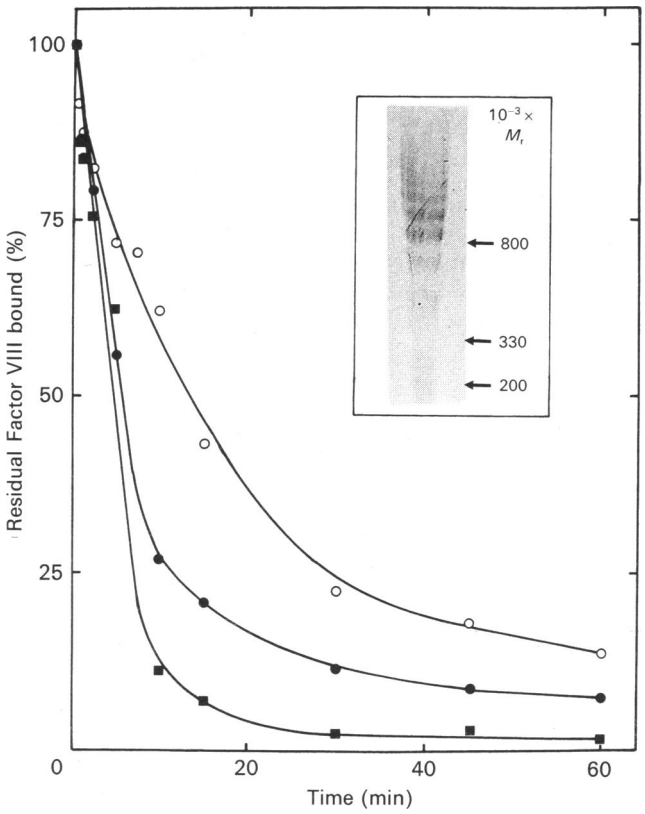

Fig. 1. Dissociation of Factor VIII from immobilized vWF

Micro-titre wells were incubated with vWF (33.5 munits/well), and Factor VIII (110 munits/well) was incubated with the immobilized vWF (3.4 munits), as described in the Materials and methods section. Subsequently the Factor VIII incubation mixture was aspirated off and 100 μl portions of buffer containing no (○), 67 munits (●) or 670 munits (■) of purified multimeric vWF in 0.2% (w/v) human serum albumin/0.1% (w/v) Tween 20 in 2.5 mM-$CaCl_2$/150 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.4, were incubated in the wells for various time intervals at 37 °C. Then the solution was aspirated off and residual Factor VIII bound to the vWF-coated wells as well as dissociated Factor VIII were quantified spectrophotometrically as described in the Materials and methods section.The inset shows the multimeric composition of the purified vWF. vWF (25 μg) was subjected to 0.1%-SDS/1%-agarose-gel electrophoresis and stained with Coomassie Brilliant Blue. The positions of the M_r markers laminin (M_r 800000), thyroglobulin (M_r 330000) and myosin (M_r 200000) are indicated.

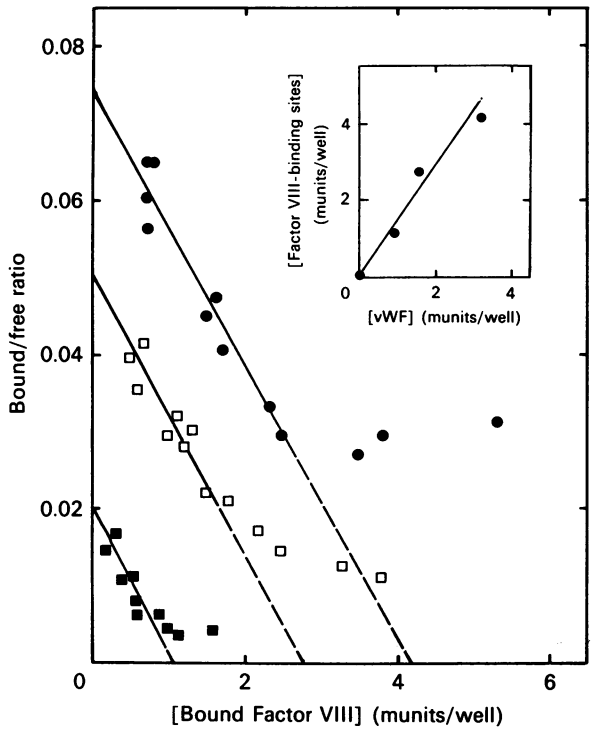

Fig. 2. Scatchard analysis of Factor VIII-binding data obtained with different amounts of vWF coating

Factor VIII was incubated in micro-titre wells that had been coated with various concentrations of vWF, and coated vWF and bound Factor VIII were quantified, as described in the Materials and methods section. The inset shows the amount of high-affinity Factor VIII-binding sites per well as a function of the amount of vWF coated.

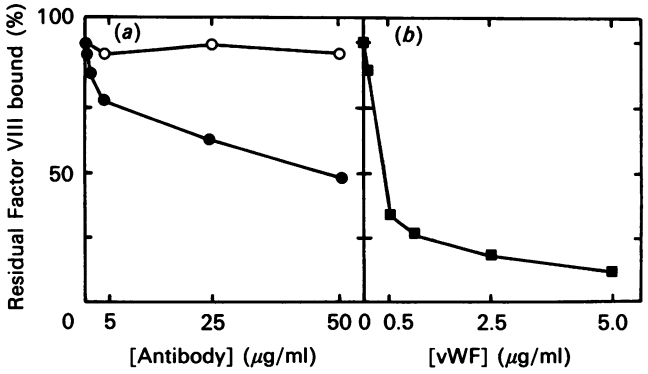

Fig. 3. Competition between vWF and monoclonal anti-(Factor VIII) antibody CLB-CAg 69 for Factor VIII binding

(*a*) Purified Factor VIII (1 unit/ml) was incubated with 0–50 μg of CLB-CAg 69/ml (●) or with the control monoclonal anti-(Factor VIII) antibody CLB-CAg 9 (○) for 2 h at 37 °C. Subsequently the vWF binding assay was performed as described in the Materials and methods section. (*b*) Purified Factor VIII (1 unit/ml) was incubated with 0–5 μg of vWF/ml (0–0.5 unit/ml) for 2 h at 37 °C. Subsequently Factor VIII binding was assayed essentially as in (*a*), except that the micro-titre wells had been incubated overnight at 4 °C with 500 ng of CLB-CAg 69/well instead of vWF.

13 min. This was consistent with the first-order rate constant of 5.4×10^{-2} min^{-1}, which was derived from a linear plot of the logarithm of the concentration of residual Factor VIII bound versus time (not shown). In the presence of vWF multimers (see Fig. 1 inset) the rate of dissociation increased in a dose-dependent manner. This demonstrates that Factor VIII binding to immobilized vWF was specific and reversible, thus permitting equilibrium binding studies. Fig. 2 shows the Scatchard analysis of binding data obtained at various amounts of vWF coated. These data suggest the presence of more than one class of binding sites. For the high-affinity sites, the dissociation constant was independent of the amount of vWF coated and was calculated assuming that the M_r and plasma concentration of Factor VIII are 260000 and 0.1 μg/ml respectively [1], to be $2.1 (\pm 0.3) \times 10^{-10}$ M (mean ± S.D., $n = 8$). The number of high-affinity binding sites per well increased linearly with the amount of vWF coated (Fig. 2 inset). Per 1 munit (or 10 ng) of vWF, the number of Factor VIII-binding sites was equivalent to 1.5 munit (or 0.15 ng). Given an M_r value of 250000 for monomeric vWF [1], the apparent Factor VIII/vWF stoichiometry is about 1:70. This value indicates that only 1–2% of the vWF subunits participates in this interaction. At high Factor VIII concentrations (higher than 1750 munits/ml, 175 munits/well) the binding data suggested the existence of additional binding sites with lower affinity. The extensive washing required in binding studies at higher Factor VIII concentrations introduced experimental variations that hampered precise characterization of the lower-affinity binding.

In order to identify the sites involved in the high-affinity Factor VIII–vWF interaction, competition studies were performed with several monoclonal antibodies against Factor VIII. One antibody against plasma Factor VIII, coded CLB-CAg 69, was found to interfere in the Factor VIII–vWF interaction (Fig. 3*a*). Similarly, vWF was an effective inhibitor of the binding of Factor VIII to the immobilized antibody (Fig. 3*b*). This indicates that the epitope of this antibody may be involved in vWF binding. Previous studies have demonstrated that the epitope of the antibody CLB-CAg 69 is located between amino acid residues 1648 and 1779 in Factor VIII [19]. Pilot experiments (results not shown) using immunoblotting of purified Factor VIII have indicated that antibody CLB-CAg 69 recognizes the Factor VIII light chain (residues 1649–2332; see Fig. 4), but not the cleavage product (residues 1690–2332) that is obtained after thrombin digestion. This provided indirect evidence that the sequence at residues 1649–1689, which represents a remarkably acidic region in Factor VIII [5], contains at least part of the epitope of the antibody that interferes with vWF binding. In agreement with this hypothesis, the antibody was found to recognize a synthetic peptide representing the sequence Lys^{1673}–Arg^{1689} (see Fig. 4).

During the course of our investigations, similar findings were reported by Foster *et al.* [23], who described that the Factor VIII–vWF binding could be blocked by an antibody against plasma Factor VIII recognizing an epitope on a synthetic peptide representing the sequence Val^{1670}–Glu^{1684}, whereas antibodies directed against adjoining sequences did not affect vWF binding. The partial overlap between the Val^{1670}–Glu^{1684} sequence and that of our peptide (see Fig. 4) suggests that the site

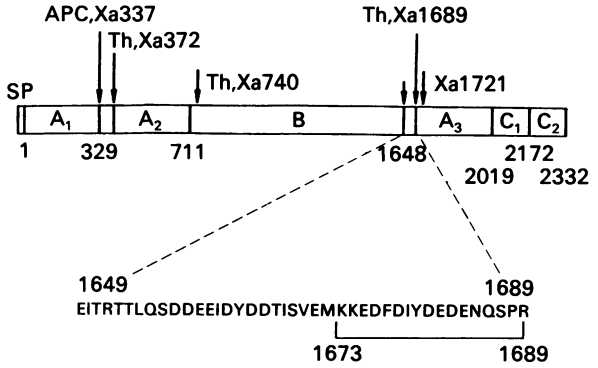

Fig. 4. Schematic representation of the Factor VIII protein and the amino acid sequence of the *N*-terminal acidic region of the light chain

At the top the protein is represented by the open bar; the signal peptide (SP) and the various domains (A_1, A_2, B, A_3, C_1, C_2) are indicated. The arrows mark the known cleavage sites for thrombin (Th), Factor Xa (Xa) and activated protein C (APC) [7]. The bottom shows the acidic region of the light chain; the bar indicates the synthetic peptide containing the epitope of the monoclonal antibody CLB-CAg 69 that inhibits vWF binding.

involved in vWF binding is located within the sequence Lys^{1673}–Glu^{1684}. Since all antibodies known so far to recognize this specific Factor VIII sequence appear to interfere in vWF binding, it seems likely that this site is directly involved in the Factor VIII–vWF interaction. This would be in agreement with recent observations that the presence of the 41-residue acidic region in the light chain is required for vWF binding [26]. Competition experiments demonstrated that the peptide Lys^{1673}–Arg^{1689} effectively inhibits the binding of the antibody CLB-CAg 69 to Factor VIII: at a peptide concentration of 1 nM, more than 90% inhibition occurred under the conditions of Fig. 3(*b*). In contrast, no inhibition of the Factor VIII–vWF interaction was found with peptide concentrations up to 0.5 μM. An explanation for this finding may be that the synthetic peptide does not reflect the complete vWF-binding site. In this regard, it may be noted that this sequence contains a tyrosine residue (Tyr^{1680}) that is sulphated as a post-translational modification of the Factor VIII protein [27]. Sulphation of the Tyr^{1680} residue may be essential for vWF binding.

With regard to the stoichiometry within the Factor VIII–vWF complex, it is of interest to know the precise localization of the sites of interaction. In contrast with the site on Factor VIII, the binding site on vWF has not been characterized in detail. This Factor VIII-binding site has recently been assigned to the *N*-terminal 272 amino acid residues of the mature vWF subunit [15]. If this site is accessible on all subunits of multimeric vWF, a 1:1 stoichiometry of Factor VIII–vWF binding would be conceivable. Indeed, 1:1 binding has been observed in sedimentation-velocity studies for the pig proteins [28]. However, it is not evident from such studies whether or no the known heterogeneity of vWF introduces multiple binding affinities. For the human proteins, data have been reported suggesting that the Factor VIII/vWF stoichiometry may be as low as 1:25 [29], a value that approximates the Factor VIII/vWF ratio in human plasma [1]. In our studies the high-affinity interaction was restricted to about 1–2% of the subunits of the vWF multimers. The apparent existence of additional binding sites with lower affinity may be explained by heterogeneity in exposition of the Factor VIII-binding site. One factor that may exert a steric effect is the presence of the vWF propeptide, which is adjacent to the Factor VIII-binding domain, and can be detected on a few per cent of the subunits of vWF multimers secreted by endothelial cells [1,14]. Alternatively, multimerization (cf. Fig. 1 inset), which involves cysteine residues located close to the Factor VIII-binding region [13,15], may affect Factor VIII-binding affinity. Further studies will be needed to assess whether or not Factor VIII-binding sites on the free termini differ from those within the vWF multimeric chains.

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